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The Effect of Feeding Various Levels of Fluoride to Dairy Cattle on Their Pepsin Activity

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THE EFFECT OF FEEDING VARIOUS LEVELS OF FLUORIDE TO DAIRY CATTLE ON THEIR PEPSIN ACTIVITY

by

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INTRODUCTION

In certain industrial areas fluorides are present in various amounts in the atmosphere. These fluorides are liberated by industrial processes at high temperature in treatment of materials containing fluoride, either as a natural impurity, or as added material for technical purposes. Equipment and procedures have been installed in many plants to reduce the output of fluorides significantly, yet forage and hay grown near the plants may contain elevated amounts of fluorides. The feeding of such material to young livestock for many months may cause fluorosis or chronic fluoride poisoning. Some springs contain a high concentration of fluorides and the continuous use of this water as drinking water by animals may cause fluorosis also.

A number of enzymes have been reported to be inhibited by fluorides. Enzymes are biological catalysts which alter the rate of chemical reactions in living tissues. Some of them bring about the breakdown of foodstuffs in the body and enable the energy so released to be used for physiologically important processes. Inhibition of enzymes may result in severe physiological dysfunction; i. e., cells may be cut off from an adequate supply of energy. During the past few years, much attention has

been given to the idea that the primary point of attack of certain toxic substances is certain enzyme systems which are vital to the normal function of the body: In other words, that the toxic property of these substances is due to their ability to poison certain physiologically essential enzymes systems specifically and in low concentration.

The effect of sodium fluoride on enzyme systems is due to the fluoride ion. The fluoride ion inhibits all enzymes which require divalent cations such as Ca^{++} and Mg^{++} for full activity. The reason for this is the ability of fluoride ion to form insoluble salt with these cations which are not catalytically active. This is the basis for the well known use of fluoride as an anti-coagulant in blood-clotting. Enzymes which require divalent cations as activators are rather widespread and serve many important functions, one extremely important class are those which are concerned with the transfer of phosphate groups.

One of the enzymes inhibited by fluoride in-vitro is pepsin. Inhibition of an enzyme such as pepsin may result in an organism being deprived of an adequate supply of energy. Pepsin is important in the digestion of proteins. Therefore, the study of the effect of feeding controlled amounts of fluoride to dairy cattle on the activity of pepsin is of interest.

The objective of this investigation was to determine and compare the enzymatic activity level of pepsin or its

precursor extracted from the abomasum of two groups of Holstein heifers and cows which had received various amounts of fluorides in their diets over varying periods of time.

REVIEW OF LITERATURE

Tauber ('49) reported that the protein digestion power of gastric juice was first observed in 1836 by Schwann who called the enzyme responsible for this effect pepsin.

Langley found that gastric mucosa contained a substance which was not changed by alkali, as is pepsin, but it was transformed into pepsin when the solution was acidified. He considered it to be an inactive precursor of pepsin called "pro-pepsin" or "pepsinogen" (Herriot '38a). The question of the activity of pepsinogen is complicated by the fact that pepsinogen is unstable in acid solution and pepsin is active only in acid solution and it is difficult to determine whether pepsinogen is active or not. Pepsinogen and pepsin can easily be demonstrated to be two different substances because they have many properties which are different.

Properties of Pepsin and Pepsinogen

Pepsin has the property of clotting milk at $_{p}H$ 5 while pepsinogen has no effect on milk (Tauber, '49). Pepsin can liquify gelatin at pH 4.7 while pepsinogen is without effect (Northrop '31a). Desreux and Herriot ('39) have shown that crude pepsin preparations may contain

more than one component and that the fraction most active in hydrolyzing gelatin is inactive at pH 2, the pH at which pepsin is most active on protein substrates.

Tisolius et al. ('38) found that the isoelectric point of pepsin is about pH 2.7 with a minimum solubility near this point and the isoelectric point of pepsinogen is about 3.7.

The molecular weight of pepsin as is determined from osmatic pressure in M/1 acetate at pH 4.6 is 35,000 ± 1,000, and of pepsinogen is 42,000 ± 3,000. From the diffusion coefficient pepsin has a molecular weight of 70,000, from sedimentation data 37,000, and from one atom phosphorus per mole 34,000 (Philpot et al., '33).

Neutral, salt-free water solutions of pepsinogen may be heated to boiling and then cooled without loss of potential activity. If sodium Chloride is added to the hot solution, the pepsinogen is entirely denatured. Pepsinogen is almost completely in the native form at or below 50° C., indicating a very high heat of reaction (Northrup et al., '48). Pepsinogen is denatured by strong alkali at pH's greater than 9.0 and this reaction is reversible for some time. Both the alkali and heat reactions of pepsinogen is entirely different from those of pepsin under the same conditions, since pepsin in similar concentrations is instantly and almost irreversibly denatured by temperatures of over 70° C. or by alkali.

When pepsinogen is exposed to 50 percent ethyl alcohol for 24 hours, it is completely destroyed (Tauber, '49).

It cannot be activated with hydrochloric acid. Pepsin, however, is quite resistant under similar conditions.

Fenger and Andrew (3) pointed out the purified enzyme, pepsin, is a nearly ash-free protein, slightly soluble in distilled water, but clearly and completely soluble in acidified water.

Michaelis et al. and Ringer reported that most samples of pepsin, as obtained from gastric juice, migrated always to the anode, even in strongly acid solutions. The addition of protein split products, however, caused the enzyme to migrate to the cathode on the acid side of about $_{p}$H 3.0. Ringer concluded that the enzyme was an acid (Herriott et al. ('40). This result was confirmed by Northrop ('25) that pepsin was a negatively charged monovalent acid at least as far as $_{p}$H 1.0.

The fact that pepsin contains at least two free primary amino groups but does not become positively charged even in 0.1 M acid indicates the presence of a very strong acid group. The amino acid content does not indicate the presence of any unusual organic acid group. The pepsin molecule contains one atom of phosphorus and the acid group may be that of phosphoric acid.

Pepsinogen has been isolated in crystalline form. It is a colorless protein crystallizing in very fine needles from slightly more than 0.4 saturated ammonium sulfate solution at $_{p}$H 5.2-5.6 (Herriott, '38a).

The first enzyme to be prepared in crystalline form was pepsin which was crystallized by Northrop ('33).

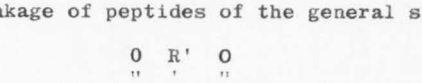
Fractionation of bovine gastric juice by various salt solutions resulted in the protease activity being concentrated in a protein fraction. This fraction also contained a large amount of a mucilaginous substance which made the precipitates hard to work with. Fenger, Andrew, and Ralston ('28) found that the mucilaginous impurity could be precipitated from solution by cold 75% acetone with a loss of nearly half of the protease activity. To prepare crystalline enzymes many recrystallization and purification steps are necessary and the resulting product may represent only a few percent of the initial activity.

When solutions of pepsinogen are made more acid than $_{p}H$ 6.0, pepsinogen is converted into pepsin. The conversion of pepsinogen into pepsin is an autocatalytic reaction at $_{p}H$ 4.6. The conversion has a maximum rate near $_{p}H$ 2.0. The addition of pepsin or increasing the concentration of pepsinogen increases the rate of conversion under all conditions.

When pepsinogen is autocatalytically converted into pepsin there is a simultaneous production of non-protein nitrogen (nitrogen not precipated by 2.5% CCl_3COOH) to the extent of 15-20% of the pepsinogen nitrogen. With ordinary proteins, the action of pepsin results in the hydrolysis of the protein into a number of smaller molecules so that the substrate protein is completely destroyed. Part, if not all, of the 15-20% of the non-protein nitrogen after being produced as a result of conversion of pepsinogen into pepsin, combines with pepsin between $_{p}H$ 5.0-6.0 to

form a dissociable inhibitor-pepsin complex. The inhibitor complex dissociates upon dilution and upon acidification and is destroyed by pepsin between $_{p}H$ 2-5. Calculations of the molecular weight of the inhibitor from N-dinitrophenyl end-group analysis and the amino-acid analysis led to values of 3100 and 3242, respectively. A relatively high lysine content was found and leucine was identified as the terminal amino acid (Van Vanakis et al. '56).

Pepsin's proteolytic activity appears to be confined to peptide linkage of peptides of the general structure.



The attack on the peptide is at the position indicated by the arrow if the following conditions are met (Laider, '58). 1. The NH-CHR'-CO part of the molecule must correspond to an -amino dicarboxylic acid of L- configuration and the second COOH of the amino acid must be free. 2. The NHR" part of the molecule must correspond to an aromatic L-amino acid such as tyrosine or phenylalanine. The COOH is the part of the molecule need not be free but an NH_2 group must not be present. 3. The RCO part may be anything as long as there are no free NH_2 groups in the vicinity of the linkage to be hydrolyzed.

Site and Source of Pepsinogen Secretion in Bovine

The bovine has a ruminant stomach which is characterized anatomically by its great size and its division into four distinct compartments, designated as rumen or

first stomach, reticulum or second stomach, omasum or third stomach and abomasum or fourth stomach. The latter stomach is a true stomach in which the digestion takes place. The gastric glands which are located in the mucosa membrane of the abomasum are connected to its surface by numerous orifices. The gland cells are of three main kinds; body chief cells, neck chief cells and parietal cells. The body chief cells are undoubtedly the enzyme producers. Their so-called zymogen granules are generally assumed to be the substance from which enzymes are derived, although definite proof is lacking. The neck chief cells line the upper part of the gastric glands. They are believed to be mucus-secreting cells. The parietal cells produce the hydrochloric acid of the gastric juice. As a part of the organic matter of gastric juice at least three enzymes are found; pepsin, rennin, and gastric lipase. Pepsin is a proteolytic enzyme whose precursor pepsinogen is formed in the body chief cells of the gastric glands. Pepsin is activated in the stomach by hydrochloric acid. The optimum pH for the action of pepsin varies somewhat with different proteins and ranges from approximately pH 1.5 to 3. Pepsin in this favorable acid medium converts proteins into simpler products as proteoses and peptones. The secretion of gastric juice is a continuous process but the rate of secretion increases when food is eaten (Dukes, '47).

The potential proteolytic enzymatic material which can be extracted from the mucosa of the abomasum is

pepsinogen but the enzymatic activity can only be measured in terms of the pepsin obtained from the pepsinogen.

Effect of Fluorides on Pepsinogen

Razenkov ('26) reported that sodium fluoride produced a greater secretion of gastric juice in dogs while Gaucher ('09) found that when sodium fluoride was added to milk the secretion of gastric juice in the dog fed the milk was retarded. Clifford ('28) found that the clotting of milk by pepsin could be inhibited by adding soluble fluorides above 0.02 M concentrations.

METHODS OF PROCEDURE

Experimental Design

One group of 16 dairy heifers and one group of 32 dairy cows employed in this study were part of a larger project on the effects of fluoride at the Utah Agricultural Experiment Station.[1]

Preventive medical procedures were practiced in detail to eliminate in so far as possible, the possibility of any disease interfering with or altering the experimental program and results. Clinical examinations and observations were made every fourteen days during the experiment and recorded on IBM cards.

The tissues used in this study were obtained from the animals on which detailed studies on the effects of feeding fluorides had been conducted for several years at the Utah Agricultural Experiment Station. The tissues and data from animals receiving normal treatment were used as a standard to compare and evaluate the response of the experimental animals to their respective fluoride intake. Records were kept on the individual hay and grain consumption of each animal on a daily and two-week basis. Hay was fed on a free choice basis.

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[1]Unpublished information in the files of the Utah Agricultural Experiment Station.

At the time of necropsy, internal organs were weighed and measured. Tissues were taken for chemical analysis, enzyme studies, x-ray, and histopathological evaluation.

The sixteen heifer experiment

The heifers weighed from 502 to 603 pounds at the beginning of the experiment and were assigned at random to the treatments in Table 1.

Table 1. Design of sixteen heifer experiment

Treatment	Animal number			
1. Alfalfa hay with low fluoride residue	64	65	48	55
2. Alfalfa hay with elevated fluoride residue	62	49	59	61
3. Alfalfa hay with fluoride residue plus calcium fluoride to give a fluoride residue equivalent to alfalfa hay with elevated fluoride residue	53	58	56	47
4. Alfalfa hay with low fluoride residue plus sodium fluoride to give a fluoride residue equivalent to alfalfa hay with elevated fluoride residue	51	57	50	54

Heifers were purchased for uniformity and health. They were approximately six months of age at the start of the experiment. All the heifers were purchased in areas

where vegetation fluoride residue levels were not above normal.

Heifers were housed and cared for at the Utah State University veterinary science animal research unit on the Logan campus. Special facilities were constructed for this purpose. Alfalfa hay with elevated fluoride residue was purchased in Utah County on the basis of quality and fluoride residue. Alfalfa hay with low fluoride residue was purchased from an area not subjected to elevated levels of air-borne fluoride. The low fluoride hay had an average fluoride residue of 17 ppm and the high fluoride hay had an average fluoride residue of 82 ppm. Hay of the two required specifications was as nearly uniform in quality as could be obtained.

Each animal was fed two pounds of grain. Fluorine in the form of calcium fluoride or sodium fluoride was added to the grain mixtures. Combinations of the grain mixtures were used to give the desired levels of fluorine. The amounts of calcium fluoride and sodium fluoride in the grain mixtures for each heifer, on treatments three and four (Table 1), were adjusted every two weeks to maintain the fluoride intake approximately equal, on a ppm dry weight feed intake basis, to that ingested by the heifers on treatment two that received the high fluoride alfalfa hay. The hay was analyzed at frequent intervals to facilitate adjustments for fluoride intake.

Liver and kidney function tests and metabolism trials were conducted on each heifer near the end of the experiment, which lasted about twenty months.

The average mg. of fluorine per kg. of body weight received by the animals over the entire experiment was: 0.20 for low F^- hay, 1.14 for high F^- hay, 1.32 for low F^- hay + CaF_2 and 1.29 for low F^- hay + NaF. This corresponded to 11, 62, 69, and 68 ppm of fluoride in the dry matter consumed, respectively.

The only animal to show definite lameness was animal number 64, which was one of the control group receiving the non-elevated fluoride ration. She was injured during the metabolism trials and remained severely lame in the right front leg until necropsied.

The thirty-two cow experiment

These animals are referred to as cows in this thesis because of their age at the time of the end of the experiment. Special facilities were constructed for housing and care of these cows at the Utah State University dairy farm on Logan campus.

The animals were three to four months of age and weighed from 180 to 274 pounds at the beginning of the experiment.[1] They were segregated according to weight and divided into two replications of 16 animals each. The 16 largest animals were allotted at random in replication one and the 16 smallest animals were allotted to the treatments in replication two, as outlined in Table 2.

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[1]Unpublished information in the files of the Utah Agricultural Experiment Station.

Table 2. Design of thirty-two cow experiment

Percent defluorinated calcium phosphate in grain	Grain lbs/day	Animal number included in study			
		Fluoride levels of diet (ppm)			
		Control 8-10	25	50	100
Replication 1					
1	2	2	3	1	X
3		13	33	7	10
1	4	6	16	5	X
3		4	9	15	14
Replication 2					
1	2	29	23	22	21
3		19	27	31	25
1	4	20	30	17	18
3		32	24	20	X

X-animals deceased before this phase of experiment was started.

Samples of feeds going into the grain mixture and all grain mixtures were taken for chemical analysis whenever changes were made. The hay was sampled every two weeks and a composite sample was analyzed for each period. The treatments consisted of four levels of fluorine (basal diet, 25, 50, and 100 ppm based on hay intake), two levels of calcium and phosphorus (one percent and three percent defluorinated calcium phosphate in the grain mixture), and two levels of grain (two and four pounds daily).

Alfalfa hay was fed ad libitum. At the end of each two-week period the amount of hay consumed was calculated and approximate amounts of fluorine were fed in a pelleted grain mixture to give the desired fluorine level in relation to the hay intake.

When the diets were analyzed the fluorine content based on the moisture-free hay consumption was as follows: 11, 28, 56, and 109 ppm, respectively. On a total diet basis (hay plus grain) the values were 10, 24, 48, and 96 ppm, respectively. On a per kg. of body weight basis the values were 0.32, 0.62, 1.12, and 2.12 mg. respectively.

The cows were kept on experiment for about seven years.

Three cows which were all from the 100 ppm fluoride treatment died during the experiment, but the cause of death has not been determined.

Preparation of Tissue

Obtaining tissue from animal

When the experimental animals were sacrificed only one animal was slaughtered per day so that all samples obtained from the animal could be properly handled. Soon after the abdomen of the cow was opened, the fourth stomach was detached and slit lengthwise. The contents of the fourth stomach were emptied and the lining of the stomach was washed well with cold water. The lining was inspected for gross abnormalities and a section of the lining from

the middle third of the abomasum was quick frozen in a plastic carton and stored six months to one year.

Thawing

The frozen tissue samples were taken from the freezer and placed in the refrigerator for about thirty hours to thaw. Then the tissues were brought out of the refrigerator and left at room temperature for almost four hours to be completely thawed out.

Obtaining of mucosa

Mucin was removed from the inner surface of the abomasum by gently scraping with the sharp edge of a knife and then washing in a stream of distilled water. Excess moisture was removed by drying the tissue between paper towels. The mucosa layer was scraped from different spots of the abomasum so that a representative sample could be obtained.

Focal areas showing abnormalities were observed in some abomasum in which the mucosal surface was slightly elevated. The color of these areas was pale-yellow to yellow-green and readily discernable from the remainder of the pink, normal appearing mucosal surface.

Storage of mucosa

The scraped mucosa of each abomasum was well mixed, put in a covered aluminum container, and frozen until the enzyme extract could be prepared.

Extraction of Enzymes

Grinding

One gram of the mucosa was weighed in a 50 ml. plastic tube 30 x 100 mm. which fits the Lourdes Multi-Mixer as well as the small head of the Lourdes model L R high speed refrigerated centrifuge. About 10 ml. of distilled water was added to the weighed sample and it was ground in the Multi-Mixer for exactly ten minutes. The grinder was attached to an automatic timer. During the grinding of mucosa, the plastic tube containing the mucosa was kept in ice water to remove the heat which is produced during the grinding.

Ammonium sulfate extraction of pepsinogen

The enzyme extract was prepared from bovine abomasum by the method described by Colowick and Kaplin ('55). The mucosa was extracted with saturated ammonium sulfate in sodium bicarbonate and then filtered with the aid of Filter-cell and Hyflo Super-cell.

Enzyme extracts from the abomasums of the 16 heifers were prepared according to this method and analyzed for pepsin activity. The data which was obtained was not reliable. Analysis of different preparations of the same mucosa gave values ranging from none to several different levels of enzymatic activity. Difficulties were encountered in the filtration process which took over an hour.

Comparison of water extraction and ammonium sulfate extraction of pepsinogen

In order to obtain a more reliable extraction of the enzymatic activity, water extraction was tried and results compared with those from the ammonium sulfate extraction.

Equal sized samples of ground mucosa were mixed with 3 ml. of 0.6 saturated ammonium sulfate in 0.13 M $NaHCO_3$, and with 3 ml. of distilled water. Both mixtures were stirred at room temperature for an hour with an electrical stirrer, and centrifuged in a Lourdes high speed refrigerated centrifuge at 10,300 X G. for ten minutes, filtered through filter paper, Whatman No. 4, and diluted to 25 ml. with distilled water.

The centrifuged residue of each preparation was mixed with distilled water, ground, stirred, centrifuged, filtered and diluted to 25 ml. as had been done with initial mucosa. These preparations were called ammonium sulfate extract, water extract, ammonium sulfate residue extract, and water residue extract.

All of the preparations underwent the chemical analysis for pepsin enzymatic activity with the hemoglobin procedure under standard conditions. Each sample was determined in duplicate. In Table 3 is represented the average optical density of each preparation.

The results of these experiments demonstrate that saturated ammonium sulfate precipitates most of the enzyme and the enzyme precursor molecules along with the other particles which have been discarded. Moreover, the

Table 3. The enzymatic activity in ammonium sulfate and water extracts and their residues

Preparation	Volume of extract in ml.*	Blank	Optical density
Extraction with .45 saturated ammonium sulfate in .1M sodium bicarbonate	25	0.410	0.101
Extraction with distilled water	25	0.435	0.800
Residue of extraction with .45 saturated ammonium sulfate, .1M Sodium bicarbonate, extracted with distilled water.	25	0.380	1.000
Residue of extraction with distilled water, extracted with distilled water.	25	0.380	0.142

*One gram of ground mucosa was diluted to 25 ml. with distilled water

relative value of the loss of the enzyme and the precursor or the presence of them cannot be estimated since there is no way of measuring them. Therefore, the procedure of preparing the pepsinogen extract with ammonium sulfate was not used.

Water extraction of pepsinogen

The following procedure was developed for extracting pepsinogen with water. It simplified the process and gave reproducible results.

Almost 20 ml. of distilled water was added to one gram of ground mucosa. The mixture was placed in the refrigerator at 5° C. for about twenty hours to obtain complete extraction. The samples were centrifuged for fifteen minutes at 10,300 X G. in the high speed refrigerated centrifuge. The filtrate was poured into a 100 ml. volumetric flask and brought up to volume with distilled water. The diluted enzyme extract was transferred into a 250 ml. plastic bottle and stored in the freezer until analysis could be completed.

Enzymatic activity in repeated extractions of mucosa

Different samples (1 gram each) of mucosa from the same abomasum were taken to determine the relative amounts of enzyme extracted from the cells of mucosa in sequence extraction. The filtrate of each extracted sample was diluted to 50 ml. and named first extracts. The residues of the above filtrates were mixed with distilled water, ground, and centrifuged again. Their filtrates were also

diluted to 50 ml. and were called second extracts. The residues which were left from the second extracts were mixed with distilled water, ground, and centrifuged once more and the filtrates were diluted to 25 ml. These latter extracts were called third extracts. Also in other preparations, concentrated first and second extracts of the mucosa were combined and diluted to 50 ml. and to 100 ml. volumes.

These extracts were assayed for pepsin activity. The relative concentration of enzyme molecules extracted in the different steps of extractions were demonstrated by their average optical densities as shown in Table 4.

This experiment shows that all of the enzyme present in the cells of mucosa was extracted by the second extraction. Comparison of the extract mixtures of first and second extracts diluted to 50 and 100 ml. showed more enzymatic activity present in proportion to the dilution in the 100 ml. combined extracts than in the 500 ml.

Determination of Enzymatic Activity Level of Pepsin

In the hemoglobin method for the estimation of proteinase, hemoglobin is digested under standard conditions, the undigested hemoglobin is precipitated with trichloracetic acid, and the amount of unprecipitated protein split products, which is a measure of the amount of proteinase present, is estimated with the phenol reagent which gives a blue color with tyrosine and tryptophane.

Table 4. Completeness of enzyme extraction in distilled water

Extracts	Volume of extract	Optical density
1st extract	50	0.585
2nd extract	50	0.005
3rd extract	25	0.000
1st and 2nd extract	50	0.630
1st and 2nd extract	100	0.400

Hemoglobin substrate

The method used in this assay is a modification of Anson's procedure ('37) for the estimation of proteinases with hemoglobin. A powdered hemoglobin substrate, lyophilized and salt free, prepared by Mann Research Laboratory, was used to make a hemoglobin water solution containing 2.5 grams of protein per 100 ml. As a preservative, 2.5 mg. of merthiolate (Lilly) was added 100 ml. of the hemoglobin solution. In making up the hemoglobin solution, the foam which forms was broken with the use of a glass rod. The hemoglobin solution then was centrifuged at 2,000 R.P.M. for fifteen minutes in an International model 2 centrifuge and stored in the refrigerator until used.

Several experiments were run with various amounts of hemoglobin substrate for different concentrations of purified enzyme pepsin and different concentrations of pepsin in water extracts. All these experiments showed that hemoglobin is a very proper and reproducible substrate for pepsin whether 3, 4, or 5 ml. or 2.5 percent hemoglobin solution is used for the pepsin concentration ranges up to 0.1 mg./ml.

Tyrosine standard

A stock solution of tyrosine was prepared by dissolving 9.06 mg. of l-tyrosine in 0.2N HCL to which 2.7 ml. of 37 percent formaldehyde was added. It was diluted to the mark of a 250 ml. volumetric flask and the solution shaken well.

This stock solution contained 1 μM tyrosine per 5 ml. It was diluted as follows: 10 ml., 20 ml., 30 ml., 40 ml., 50 ml., and 60 ml. of stock solution were diluted to 100 ml. and 35 ml. diluted to 50 ml. to give concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 μM tyrosine/5ml.

A standard curve was prepared from the diluted tyrosine solutions containing from 0.1 to 0.7 μM of tyrosine per 5 ml. To 5 ml. of each solution was added 10 ml. of 0.5 N NaOH. The solutions were mixed well and 3 ml. of the phenol reagent[1] was added. After the ten minutes of color development the solutions were transferred from the test tubes to 19 x 105 mm. cuvettes. A Coleman Universal spectrophotometer was used to read the optical densities of the standards against their own blanks. The optical density readings were taken at 750 mμ. The value for the tyrosine standards when plotted gave a straight line (Figure 1).

In most runs of the pepsin assay, two different concentrations of tyrosine standard solutions were run to check the conditions of the experiment.

Analytical procedures

The hemoglobin solution and enzyme extracts were removed from the refrigerator and placed in a container of water to bring their temperature up to 25° C. Four ml. of the hemoglobin solution was put in a 30 x 100 mm. test tube

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[1]1:2 Phenol reagent-One portion of phenol reagent (Folin-Ciocalteau, '27) prepared by Fisher Scientific Co. was diluted with two portions of distilled water.

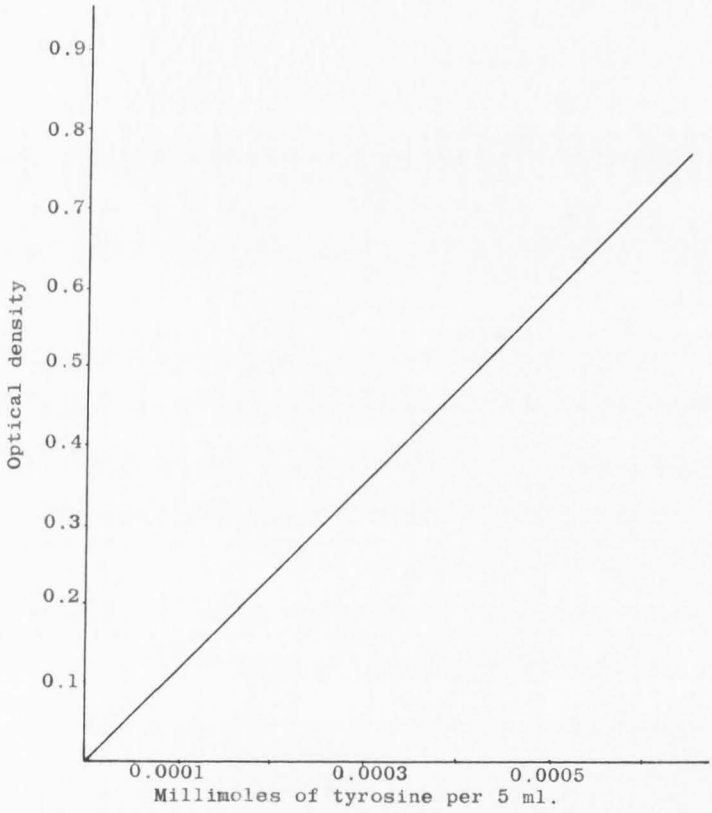

Figure 1. Tyrosine standard curve

and 1 ml. of .3 N HCl was added. Then the solution was shaken well. The $_{p}H$ of the substrate was about 1.8 (as measured with Hydrion indicator paper).

One ml. of the enzyme extract was added to the substrate and was shaken. The tube was placed at 25° C. in a water bath for exactly ten minutes. Digestion was stopped by adding 10 ml. of trichloroacetic acid (CCl_3COOH) which precipitated all of the undigested substrate and enzymes. The tube was shaken vigorously. The suspension was filtered through Whatman No. 4 filter paper into a 50 ml. Erlenmeyer flask. To 5 ml. of the filterate in an 32 x 160 mm. tube, 10 ml. of 0.5 N NaOH was added and shaken well. To this mixture 3 ml. of the phenol reagent was added. A particular pipette with a small tip was used throughout the experiment. The rate of phenol addition was the same for each sample since the end of the pipette was left free hence, the phenol was added as rapidly as it could flow from the pipette. During this addition the test tube is shaken as vigorously as possible. The time for color development was standardized at ten minutes. All the samples were run in duplicate.

A blank was run for each sample. It was prepared in the same way as the sample except the CCl_3COOH was added before the addition of the enzyme.

The units of pepsin activity in the enzyme extract samples were calculated by use of the tyrosine standard curve. The optical density reading for the 0.0005 μM of tyrosine per 5 ml., was 0.600. A constant, K, was obtained

by dividing these values, 0.005/0.600, K equals 5/6000. This constant was multiplied by the optical density reading of the extracted enzyme sample to give the equivalent of millimoles of tyrosine in the portion of 1 ml. from the original solution per ten minutes. Multiplying by the dilution factor gave the units of pepsin per ml. of extract. Thus the units of pepsin present in 1 ml. of the original extract would be optical density x 5/6000 x 16/5 x 1/10 = units of pepsin per ml. of enzyme extract or O. D. x 0.000267 = pepsin units per ml. of enzyme extract.

Nitrogen determination

In 20 x 150 mm. test tubes were placed 1 ml. of original enzyme extract and 1 ml. of the digestion mixture (50 ml. of concentrated H_2SO_4 added to 50 ml. of distilled water to which 1.2 grams of H_2SeO_3, i.e., 1 percent on the basis of SeO_2, were added). The tubes were shaken well.

To every tube two small boiling chips (Hengar granules) were added to prevent the bumping of the acid mixture upon heating. All the tubes were set in a sand bath in a slanting position. The digestion was carried out with a rather high temperature on a hot plate and was continued until the solution was clear which took about three hours. After the digestion was completed, the test tubes were set out at room temperature to cool. The digested solution was diluted with some distilled water and transferred into a 50 ml. volumetric flask. The tubes were rinsed with two

more portions of distilled water which were added to the flask. The dilution was brought to the mark and shaken well.

Seven ml. of the diluted digested solution were pipetted into 19 x 105 cuvettes. Three ml. of the Nessler's reagent[1] was added to it and shaken well.

Optical density was read against the blank prepared in the same way as the sample, after ten minutes in a Coleman Universal spectrophotometer at 430 mμ. All the samples were run in duplicate.

Nitrogen standard

A stock solution containing 250 μM of tyrosine nitrogen per ml. of the solution was made by dissolving 4.55 grams of -tyrosine in a small amount of concentrated HCl. It was then made up to 100 ml. with distilled water. This stock solution was diluted as follows: 1.25 ml., 2.50 ml., 5.0 ml., 7.5 ml., 12.5 ml., 15.0 ml and 20.0 ml. of the stock solution were diluted to 2.5 ml. to give concentration of 12.5, 25, 50, 75, 100, 125, and 200 μM/ml. of tyrosine nitrogen, respectively. For the analyses of these solutions, 0.5 ml. of each solution was diluted to

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[1]Nessler's reagent-Formula of Bock and Benedict (Hawk, et. al., '54). One hundred grams of mercuric iodide and 70 grams potassium iodide were dissolved in about 400 ml. water in a liter volumetric flask. One hundred grams of NaOH was dissolved in about 500 ml. water. After cooling thoroughly, this solution was added with constant shaking to the mixture in the flask; then made up with water to the liter mark. This solution usually became perfectly clear. When the small amount of brownish-red precipitate had settled out, the superatant fluid was ready to use.

10 ml. from which 0.5 ml. was digested, made up to 100 ml. after digestion, and 2 ml. read in a final volume of 10 ml. The concentration of nitrogen in mg. of the final dilution for each solution is shown in Figure 2. These values when plotted, gave a straight line.

The nitrogen content of the enzyme extract was calculated by use of the nitrogen standard curve. The optical density reading for the 0.0007 mg. of tyrosine nitrogen per 10 ml., was 0.280. The constant, therefore, for the standard curve would be

K = .0007/.280 = 0.0025.

The nitrogen content of the enzyme extract would be optical density reading of the sample times the constant times the dilution factor, or optical density x .0025 x 50/7 = O. D. x .01786 = mg. of nitrogen per ml. of original enzyme extract.

Phosphorus determination

To digest the enzyme extract for the phosphorus determination, 50 ml. of enzyme extract was placed in a 250 ml. beaker and 15 ml. of concentrated nitric acid was added to it. The solution was boiled gently on a hot plate until the nitric acid was almost all evaporated. Heating to the dryness was avoided. Approximately 5 ml. of a 1 to 1 mixture of concentrated nitric acid and perchloric acid was added and evaporated nearly to dryness. Then, 5 ml. of 70 percent perchloric acid was added and heated slowly to dryness.

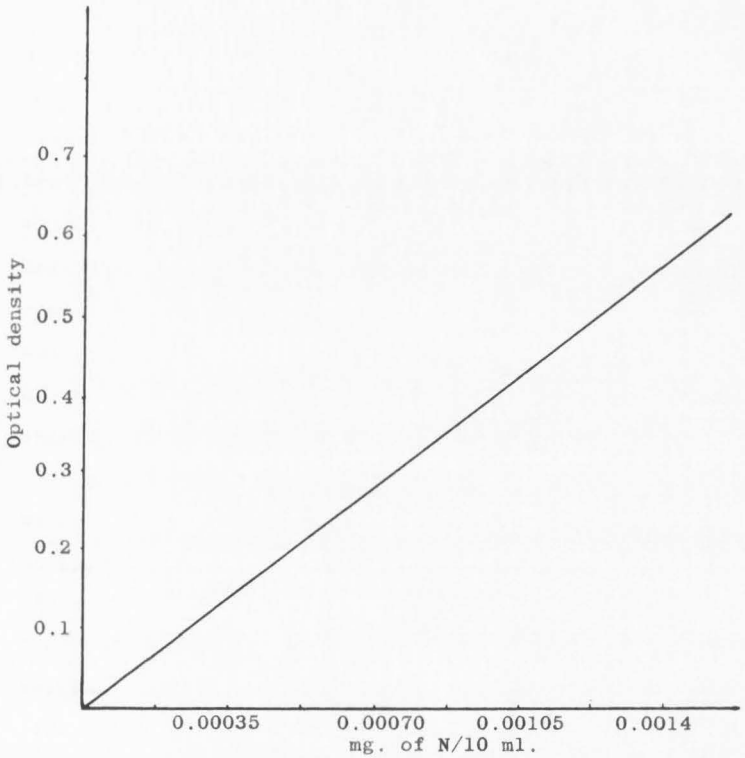

Figure 2. Nitrogen standard curve

The acid solution should be colorless or a pale yellow. If the solution was dark or undigested material is detected another portion of perchloric acid should be added and evaporated to dryness. It was necessary to remove all of the acid so that it would not interfere with the phosphorus determination but in doing so unreactive pyrophosphates were formed. The pyrophosphates were hydrolyzed to ortho-phosphate by adding approximately 20 ml. of distilled water to the cooled beaker and gently boiling the solution for two hours. Water was added as necessary to replenish water lost by evaporation. The solution was allowed to cool slightly and was diluted to 25 ml. in a volumetric flask. The solution was mixed thoroughly and placed in a labeled bottle.

A modification of the method of Chen, Tornbara, and Warner ('56) was used in the determination of the total phosphorus. One ml. portions of the digested enzyme extract was placed in each of three 20 x 150 mm. test tubes. Then 3 ml. of distilled water and 4 ml. of freshly prepared reagent "D"[1] was added to each tube. A blank was prepared from 4 ml. of distilled water and 4 ml. of reagent "D." The contents of the tubes were mixed by shaking. The tubes were placed in a rack in a water bath at 37° C. for two hours. The rack and the tubes were then removed from the

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[1]Reagent (D) must be prepared fresh for each set of determinations. One volume distilled water, 1 volume 6 N H_2SO_4, and 1 volume 2.5 percent ammonium molybdate were mixed. Then 1 volume 10 percent ascorbic acid was added.

bath and allowed to cool at room temperature (about 30 minutes). The contents of the tubes were transferred into the 19 x 105 mm. cuvettes and compared with the blank at 820 mμ in a Coleman Universal spectrophotometer. The optical density as recorded for each sample, was the average value, obtained from the three values of each sample.

The following method was used to determine the inorganic phosphorus content of the enzyme extract. Two ml. of enzyme extract was placed in a 30 x 100 mm. plastic centrifuge tube. Three ml. of 15 percent trichloroacetic acid was added to precipitate all the organic material and mixed well. It was centrifuged in an International centrifuge at 2000 RPM. Four ml. of filtrate was pipetted into a 19 x 105 mm. cuvette. Four ml. of the reagent "D" was added to it. The blank is made of 4 ml. distilled water plus 4 ml. of reagent "D." The sample is run in triplicate and the color value is compared with the blank in a Coleman Universal spectrophotometer at 820 mμ .

RESULTS AND DISCUSSION

Enzymatic Activity Unit of Pepsin

One unit of proteinase is defined as the amount of enzyme which digests hemoglobin, under the standard conditions of pH and temperature employed, at an initial rate as is liberated per minute in the digestion mixture, an amount of split products not precipitated by trichloroacetic acid which yields the same color with the phenol reagent as one millimole of tyrosine does. The specific activity of enzyme is the activity per milligram of enzyme nitrogen.

Pepsin Activity of Enzyme Extracts

The findings in the 16 heifer experiment are presented in Table 5. Although three groups of these animals had similar intakes of fluoride while the fourth or control group was on a low intake (11, 62, 69, and 68 ppm of fluoride in the hay dry matter), the average values for pepsin activity for the different treatments showed considerable variation. The form in which the fluoride was fed might have influenced the results. The group on the high fluoride hay (62 ppm) showed greater pepsin activity than either of the other groups which had fluorine added to their diet in the form of the calcium or sodium

Table 5. Pepsin units, nitrogen content, and pepsin activity of crude enzyme extract of abomasum of sixteen heifers

Fluoride treatment (hay dry matter basis)	Animal number	In 1 ml. of crude pepsin extract: Pepsin units x 10^6	Mg. of N x 10^6	Pepsin activity x 10^4†	Average pepsin activity
Alfalfa hay with low fluoride residue (11 ppm)	64	302	8216	367	
	65	80	5804	137	
	48	27	5215	51*	
	55	270	7465	361	229
Alfalfa hay with elevated residue (62 ppm)	62	155	4465	346	
	49	168	5269	319	
	59	164	5090	322	
	61	167	5036	331	329
Alfalfa hay with low fluoride plus calcium fluoride (69 ppm)	53	75	5679	131*	
	58	135	5233	257	
	56	149	5358	279	
	47	171	5983	285	238
Alfalfa hay with low fluoride residue plus sodium fluoride (68 ppm)	51	16	6108	26*	
	57	113	6662	170	
	50	100	5269	190	
	54	14	6608	20*	101

*The abomasums of these exhibited some 'abnormal' areas of the mucosa at the time of pepsin assay.

†The specific activity is obtained by dividing the pepsin units by the mg. of N.

Specific activity is based on the enzyme nitrogen, whereas the nitrogen values obtained in this experiment are the values of the protein nitrogen content of the extract which include the nitrogen from other proteins extracted along with the enzyme. Therefore, these values cannot be considered truly specific activity values of pepsin, but this was considered the best way to compare and evaluate the pepsin level from different animals. Since other protein nitrogen contributed to these values might be assumed nearly the same proportion in 1 ml. extract in all the samples, this evaluation and comparison might be considered reliable and convenient.

fluoride (329 vs. 238 and 101 for pepsin activity) and more activity than the group on the low fluoride hay. The pepsin activity for the low fluoride hay plus NaF group was one-third to two-thirds lower than for any other group. These findings, if the differences are real, would indicate that the difference was due to the form of fluoride which was fed. The low pepsin activity for the NaF group showed that NaF was a more active form of fluoride than CaF_2 as an inhibitor of pepsin activity.

If the low value of 51 in the 11 ppm fluoride hay group was the result of the treatment, the high values obtained on the high fluoride hay appeared to indicate that this increase in fluoride had a stimulating effect. Pepsin activity was one-third greater on the high fluoride hay than on the low fluoride hay. If this low value was due to chance or for reasons other than treatment, the average value for the other three animals on low fluoride hay did not differ greatly from the pepsin activity for the high fluoride hay group (288 vs. 329). In that case the 62 ppm level of fluoride had not influenced the pepsin activity. As in all biological experiments, differences between individual animals appeared to be high.

In the 32 cow experiment (Table 6) in which graded doses of fluoride were fed (10, 25, 49, and 94 ppm on a total dry matter basis), an analyses of variance of the data showed that differences due to treatment were significant at the 1 percent level. The linear and quadratic effects were also significant at the 1 percent level. The

Table 6. Pepsin units, nitrogen content and pepsin activity of crude enzyme extract of abomasum of thirty-two cows

Fluoride treatment (Total dry matter basis)	Animal Number	Pepsin units x 10^6	Mg. of N x 10^6	Pepsin activity x 10^4 +	Average pepsin activity
Basal diet	2	129	4733	273	
(10 ppm)	13	196	7412	264	
	6	131	5537	236	
	4	119	5000	237	
	29	199	6662	298	
	19	125	8304	151*	
	26	172	6965	246	
	32	150	4911	304	251
(25 ppm)	3	243	6072	400	
	33	174	5983	290*	
	16	275	7323	375	
	9	149	5447	271*	
	23	331	9109	343	
	27	283	7323	386	
	30	294	9198	319	
	24	171	4822	354*	342
(49 ppm)	1	91	6162	147*	
	7	247	7858	314	
	5	243	7412	327	
	15	83	6519	126*	
	22	53	5090	104*	
	31	47	5626	83*	
	17	72	5358	134*	
	20	48	4822	99	167
	10	130	5715	214	
(94 ppm)	14	40	5804	68*	
	21	11	4733	22*	
	25	19	5537	33*	
	18	20	5000	40*	75

*See footnote to Table 5.

group on the 100 ppm of fluoride had the lowest pepsin activity. It was of interest that the group average for the 25 ppm fluoride treatment was higher than that of any other group. The 25 ppm of fluoride appeared to have a stimulating affect on pepsin activity.

The decrease of enzyme activity observed from the mucosa of a number of animals appeared to be closely associated with the presence of "abnormal" areas of the mucosa. However, some animals that showed "abnormal" areas had high pepsin activity. While the "abnormal" areas appeared to occur more frequent in the higher fluoride treatments of the thirty-two cow experiment, the cause and the nature of these areas is unknown at this time. Individual variation between animals must also be considered. Further tests with more animals would be desirable.

Pepsin Assay of "Abnormal Areas" of Mucosa

In some abomasums, while scraping the mucosa, abnormal areas in various degrees of progress were observed. Some of the areas appeared almost penetrating. A comparison was made on the enzyme activity level of the different parts of the same abomasum bearing the various degrees of this abnormal phenomenon.

Three samples of mucosa from a particular abomasum were obtained. 1. From an extended abnormal area, 2. from a less severe abnormal area, and 3. from a normal area. On gross observation, these abnormal areas of the lining

of the abomasum appeared to be pale in color and rough to the touch. One gram of mucosa of each of the samples were ground, extracted, and centrifuged. The filtrates were diluted to 50 ml. and underwent chemical analysis for the pepsin activity, and also nitrogen (protein) content. The average optical density of each sample for each determination is shown in Table 7.

Table 7. Comparison assay of abnormal and normal areas of the mucosa

Preparation	Optical density for pepsin level	Optical density for nitrogen content
Extended abnormal area	.000	.300
Abnormal area	.003	.440
Normal area	.395	.390

Determination of Optimum Substrate-enzyme Concentration

An experiment was set up to determine the dilution of enzyme extract that gave the best results. From one enzyme extract different dilutions were made. One gram of mucosa was extracted and diluted to 50 ml. This original solution was diluted in order to make dilutions of 1 gm./100 ml., 1 gm./150 ml., 1 gm./200 ml., and 1 gm./250 ml. These various diluted enzyme extracts were assayed for pepsin enzymatic activity. The experiment was repeated in duplicate.

The average optical density obtained for each dilution was multiplied by a factor in order to represent the total enzymatic activity in the original solution (Figure 3).

Reproducibility of the Pepsin Assay Procedure

To determine if the extraction procedure and the pepsin assay procedure gave reproducible results, several experiments were conducted.

First, different concentrations of enzyme extracts from a particular mucosa sample were made in order to give 1 gm./ 50 ml., 1 gm./100 ml., 1 gm./150 ml., 1 gm./ 200 ml. and 1 gm./ 250 ml. A solution of 0.1 mg. per ml. of the purified pepsin was prepared. Equal volumes of the 0.1 mg. per ml. purified pepsin solution was mixed with each of the concentrations of pepsin extract. These enzyme preparations underwent the standardized chemical analysis for pepsin activity. The optical density values of each concentration was plotted against the concentration of the enzyme in the solution. The curve which was obtained was drawn on the same graph as a curve obtained from the various enzyme dilutions without the added purified pepsin (Figure 4).

Second, a series of purified pepsin solutions of various concentrations, was prepared containing 0.01 mg., 0.02 mg., 0.04 mg., 0.06 mg., 0.08 mg., and 0.10 mg. per ml. A 1 gram/50 ml. pepsin extract was made from a particular mucosa. Equal volumes of this pepsin extract

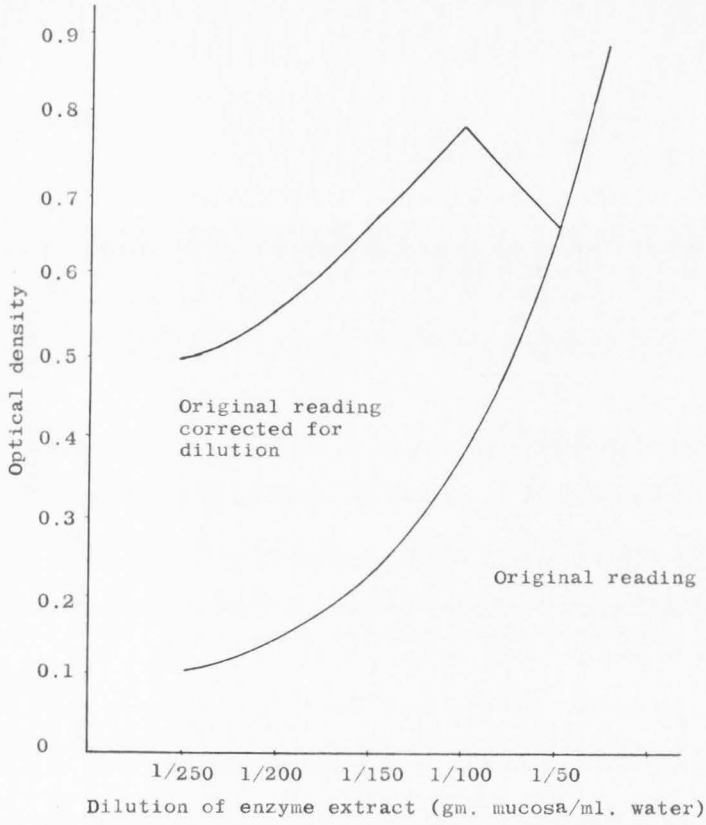

Figure 3. Enzyme activity of various enzyme-substrate concentrations

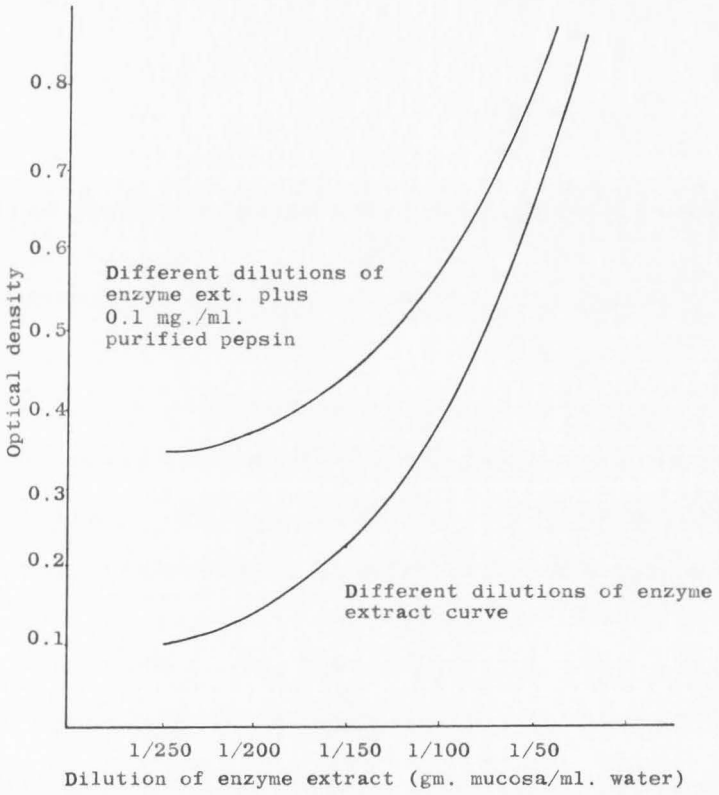

Figure 4. Enzyme extract plus constant amount of purified pepsin

were mixed with each of the purified pepsin preparations. These mixtures were assayed for pepsin activity. The optical density values of these preparations were plotted on the same graph with the pepsin standard curve. The two curves were not parallel to each other, indicating the enzyme substrate ratio was not at its optimum value.

Third, another determination was made in the same way as the second experiment. But the pepsin standard solutions were mixed equally with a 1 gm./250 ml. pepsin extract. When the optical density values of this experiment were plotted on the same graph as pepsin standard curve, it was parallel to the standard pepsin curve (Figure 5).

In the third experiment the recovery of pepsin and reproducibility of the method was evident.

Reproducibility of Results in Pepsin Assay and Protein Nitrogen Determination

New extracts from several mucosa samples were made. Pepsin assay and nitrogen content determinations on these new extracts were repeated in duplicate under standard conditions. The average optical density readings of this experiment are shown in Table 8 as are the readings obtained in previous experiments. These results show the reproducibility of the procedures employed.

Results of Phosphorus Determination

The pepsin molecule contains one atom of phosphorus per molecule, therefore, it seemed ideal to confirm the

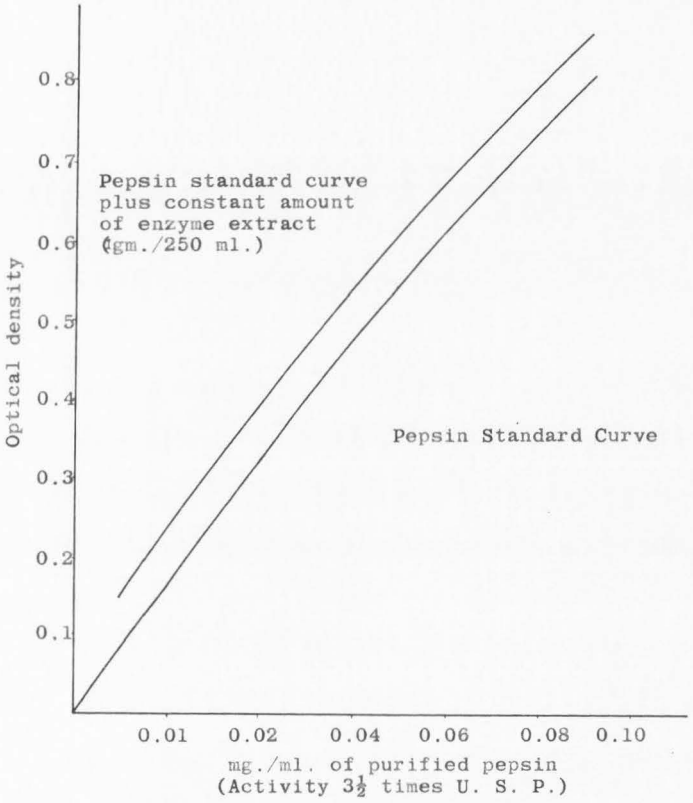

Figure 5. Pepsin standard curve plus constant amount of enzyme extract

Table 8. Reproducibility results in pepsin assay and protein nitrogen determination

Animal number	Pepsin assay		Nitrogen Determination	
	Average O.D. of previous experiments	Average O.D. of repeated experiments	Average O.D. of previous experiments	Average O.D. of repeated experiments
1	0.327	0.340	0.337	0.345
5	0.825	0.910	0.415	0.415
10	0.470	0.460	0.312	0.320
14	0.152	0.150	0.345	0.325
15	0.312	0.310	0.355	0.365
17	0.255	0.270	0.315	0.300
18	0.072	0.075	0.275	0.280
21	0.042	0.040	0.278	0.265

calculated results of pepsin enzymatic activity on the basis of phosphorus content of the enzyme extract in addition to the nitrogen content. A series of enzyme extracts were analysed for total phosphorus determination (organic plus inorganic). From the same series of enzyme extracts, another determination was made on inorganic phosphorus content. The results of these experiments demonstrate that the level of inorganic phosphorus was so high in proportion to the total phosphorus that phosphorus determination in the crude pepsin extract could not contribute to the purpose as planned. To illustrate these results briefly, Table 9 is given on the following page showing the average optical density reading values of both total phosphorus and inorganic phosphorus content of the same enzyme extracts for comparison.

Test of Fluoride Inhibition of Pepsin In-vitro

One gram of mucosa was ground with distilled water and centrifuged. The filtrate was diluted to 50 ml. with distilled water.

Different concentrations of sodium fluoride (NaF) were made to give solutions of 2 M, 0.2 M, 0.02 M, 0.002 M, and 0.0002 M.

Ten ml. of each concentration of sodium fluoride solution were mixed with enzyme extract. These preparations gave enzyme extracts of 1 gm. of mucosa in 100 ml. of 1 M, 0.1 M, 0.01 M, 0.001 M, and 0.0001 M sodium fluoride. One portion of the enzyme extract was mixed with one portion

Table 9. Comparison of total with inorganic phosphorus content of the same enzyme extract

Extract of animal no.	Average O.D. of organic plus inorganic P	Average O.D. of Inorganic P	Difference for organic P
1	0.320	0.325	-0.005
4	0.580	0.525	0.055
5	0.560	0.531	0.029
10	0.410	0.350	0.060
21	0.320	0.375	-0.055
24	0.400	0.313	0.087
25	0.360	0.300	-0.040
26	0.460	0.512	-0.052
27	0.850	0.788	0.062
31	0.360	0.287	0.073
32	0.635	0.463	0.172
33	0.850	0.888	-0.038

of distilled water as a control. These enzyme extracts were analyzed for pepsin activity. The average optical density reading of each preparation is given in Table 10.

Because considerable loss of enzymatic activity occurred in the higher sodium fluoride concentration, it was suspected that the inhibition might be due to the salting out effect of enzyme or substrate by the sodium fluoride. Therefore, the experiment was carried out using sodium chloride.

The fluoride concentrations in these tests which was found to inhibit the activity of pepsin in-vitro were far in excess of that fed to the experimental animals on whose mucosa these tests were run. Hence, the fluoride concentration in the diet would be unlikely to inhibit the pepsin in the stomach of the animal. However, the effect of daily intake of small doses of fluoride over months or years on the production of pepsinogen could not be measured.

Test of Sodium Chloride Effect on Pepsin In-vitro

Solutions of sodium chloride with the concentrations of 2 M, 0.2 M, and 0.02 M were made. Equal volumes of each of these solutions were mixed with an enzyme extract of 1 gram in 50 ml. of water. One part of the enzyme extract was diluted with an equal volume of distilled water as a control. These gave enzyme extracts of 1 gram in 100 ml. in 1 M, 0.1 M, and 0.01 M sodium chloride which were assayed for pepsin activity. In Table 11 is given

Table 10. Fluoride inhibition of pepsin in-vitro

Treatment		Optical density
Enzyme extract in	1 M NaF	0.080
Enzyme extract in	0.1 M NaF	0.480
Enzyme extract in	0.01 M NaF	0.600
Enzyme extract in	0.001 M NaF	0.620
Enzyme extract in	0.0001 M NaF	0.625
Enzyme extract in	distilled water	0.640

Table 11. Chloride activation of pepsin in-vitro

Treatment		Optical density
Enzyme extract in	1 M NaCl	0.787
Enzyme extract in	0.1 M NaCl	0.742
Enzyme extract in	0.01 M NaCl	0.700
Enzyme extract in	distilled water	0.660

the average optical density reading of the different treatments.

This experiment shows that the loss of the enzymatic activity in the previous experiment (test of fluoride inhibition of pepsin in-vitro) was due to the inhibition of pepsin by fluoride ion, not the salting out effect, and also shows that most likely the chloride ion activates pepsin in-vitro.

SUMMARY

Two groups of dairy cattle fed different amounts of fluoride in their diets were employed in this study. Sixteen Holstein heifers, divided into four animals per treatment, were fed low fluoride hay, high fluoride hay, low fluoride hay plus CaF_2 and low fluoride hay plus NaF assaying 11, 62, 69, and 68 ppm, respectively, of fluoride on a hay dry matter basis for twenty months. A group of thirty-two cows were fed four different levels of fluoride (10, 25, 49, and 94 ppm on a total dry matter basis) in their feed for seven years. Eight animals were on each treatment.

A procedure for extraction of pepsinogen from the cells of the mucosa of the middle third of the abomasum was developed. The pepsinogen was converted into pepsin and the pepsin activity and nitrogen content of the extract were determined.

The inhibition of pepsin in-vitro in concentrations greater than 0.01 M was affirmed. Chloride ion appeared to increase the enzymatic activity of pepsin in these crude extracts.

The pepsin activity values of the various groups of animals showed considerable variation as the result of the treatments.

The greatest pepsin activity was found in the group of heifers fed the high fluorine hay (62 ppm) while the group on the low fluorine hay plus sodium fluoride (68 ppm) had the lowest pepsin activity.

The highest pepsin activity was not found with the lowest fluoride intake in the 32 cow experiment but on the 25 ppm level of intake.

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